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Protein Retention in Yeast Rough Endoplasmic Reticulum: Expression and Assembly of Human Ribophorin I

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Abstract. The RER retains a specific subset of ER proteins, many of which have been shown to participate in the translocation of nascent secretory and membrane proteins. The mechanism of retention of RER specific membrane proteins is unknown. To study this phenomenon in yeast, where no RER-specific membrane proteins have yet been identified, we expressed the human RER-specific protein, ribophorin I. In all mammalian cell types examined, ribophorin I has been shown to be restricted to the membrane of the RER. Here we ascertain that yeast cells correctly target, assemble, and retain ribophorin I in their RER.

Floatation experiments demonstrated that human ribophorin I, expressed in yeast, was membrane associated. Carbonate (pH = 11) washing and Triton X-114 cloud-point precipitations of yeast microsomes indicated that ribophorin I was integrated into the membrane bilayer. Both chromatography on Con A and digestion with endoglycosidase H were used to prove that ribophorin I was glycosylated once, consistent with its expression in mammalian cells. Proteolysis of microsomal membranes and subsequent immunoblotting showed ribophorin I to have assumed the correct transmembrane topology. Sucrose gradient centrifugation studies found ribophorin I to be included only in fractions containing rough membranes and excluded from smooth ones that, on the basis of the distribution of BiP, included smooth ER. Ribosome removal from rough membranes and subsequent isopycnic centrifugation resulted in a shift in the buoyant density of the ribophorin I–containing membranes. Furthermore, the rough and density-shifted fractions were the exclusive location of protein translocation activity. Based on these studies we conclude that sequestration of membrane proteins to rough domains of ER probably occurs in a like manner in yeast and mammalian cells.

THE RER is the site of entry for nascent proteins into the secretory pathway. Both membrane and secreted proteins are recognized, targeted to the RER, and translocated into this organelle by cellular components located in both the cytosol and the ER membrane (36). Functionally characterized membrane components of the RER that participate in translocation have been shown to be largely restricted in their distribution to this membrane system (1, 10, 14, 15). The location of these proteins is maintained despite the continuity between rough and smooth ER membranes, and despite the tremendous flow of not only secretory but also membrane proteins through the RER. The question of how proteins are retained in the RER is thus a central one in the area of protein targeting and organelle biogenesis.

Retention signals have been demonstrated for luminal ER proteins from various sources. Mammalian proteins have the sequence KDEL on their COOH-termini, and the analogous sequence in yeast is predominantly HDEL (23, 28). These signals appear to interact with a membrane-bound receptor that may either retain them in the ER and/or return them should they be transported further along the secretory pathway (20, 27, 35). None of the integral ER membrane proteins, whose amino acid sequences are known, have such a signal. Moreover, KDEL seems to determine a generalized ER (both rough and smooth) localization, whereas both characterized and putative participants in translocation are restricted to rough membranes. Recent studies on membrane proteins from a variety of sources have led to the postulation that retention is based on a COOH-terminal, lysine-rich motif (24), but as with KDEL, these proteins show a generalized ER distribution, not restriction to rough domains (18). None of the proteins that have been found exclusively in the membrane of the RER contain such sequences.

Secretion and membrane biogenesis in yeast appears to be analogous to that of larger eukaryotes (25). In addition, genetic selection can be used to generate mutants defective in many aspects of these processes. It would be a tremendous advantage to be able to use yeast as a model system for studying membrane protein retention in the RER. A major stumbling block must first be overcome, however, before yeast can serve as the object of such a study. Although yeast most certainly must possess proteins that are RER-specific, none has been identified to date. Despite the rapid progress that has

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been made in identifying participants in protein translocation in yeast by genetic means (8, 9, 31, 34), none of them have yet been shown to have a uniquely RER localization. To overcome this problem for both studies on retention of RER proteins, and for cell fractionation studies leading to the isolation of purified RER membranes, we have chosen to express a "canonical" mammalian RER marker, ribophorin I, in yeast. In this way we can create a RER marker for this organism until an endogenous one can be found.

In this paper we describe the expression of ribophorin I in yeast. We find that the protein attains all of its attributes observed in mammalian cells. Most importantly, it coisolates with a ribosome-studded membrane fraction that contains the machinery for the translocation of the yeast presecretory protein prepro-α-factor. From these data we conclude that the protein retention mechanism in the RER is analogous in both yeast and mammalian cells.

Materials and Methods

Plasmid Construction

A 2,266-bp Eco RI fragment, containing the entire coding region of human ribophorin I (RI) (7), was end-filled and ligated into the yeast expression vector pEMBLyex4 at the Sma I site downstream of the 5' untranslated region of the *CYC1* gene. The construct was then transformed into a *gal1* host strain (GY41). However, although high levels of mRNA were produced, translation was totally inhibited. Translation was restored when a "GC"-rich portion of the ribophorin I 5' untranslated region immediately before the start ATG of the human RI gene was removed by site-directed mutagenesis.

Expression of Human Ribophorin I in Yeast

GY41 (4003-5B from YGSC) yeast cells (a *leu2 ade1 his4 met2 ura3 trp5 gal1 cir*$^+$) containing the pEMBLyex4-RI plasmid were grown consecutively on ura$^-$, leu$^-$ minimal plates in order to select for cells containing a high copy number of the plasmid. Starter cultures were grown to stationary phase in CA media (0.5% Bacto vitamin assay casamino acids [Difco Laboratories, Detroit, MI], with adenine, histidine, methionine, and cysteine at 50 μg/ml) supplemented with 2% synthetic dextrose. To avoid catabolite repression of the *GAL10* promoter, large cultures were grown in CA medium plus 2% ethanol to an A_{600} of 0.1, at which point galactose was added to a final concentration of 2%. Culture were then allowed to grow to an A_{600} of 1.0 before harvesting cells. Expression of the human RI gene was monitored by immunoblotting using a monoclonal anticanine RI antibody (16).

Fractionation of Yeast Cells

Separation of Membranes Derived from Rough and Smooth ER. Initial fractionation of yeast cells was performed according to the method of Rothblatt and Meyer (30). A crude supernatant was obtained by centrifugation in a rotor (model GS3; Sorvall; E. I. duPont de Nemours & Co., Burbank, CA) at 8,000 rpm for 15 min at 4°C. Aliquots of the supernatant were layered over a continuous sucrose gradient of 30–48% (wt/wt) sucrose containing LSB (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl) and centrifuged in an SW 41 rotor Beckman Instruments, Inc., Palo Alto, CA at 40,000 rpm for 18 h, at 4°C. Fractions were then collected using an Auto Densi-FLOW IIC, automatic fraction collector (Haake Buchler Instruments, Inc., Fort Lee, NJ).

Density Perturbation of RER-derived Membranes. Crude microsomal membranes were prepared from yeast containing expressed human ribophorin I according to the method of Rothblatt and Meyer (30). To observe the effect of ribosome removal on membranes containing Ribophorin I, 200 mU (A_{280}) were resuspended in either dissociation buffer containing 0.25 M sucrose, 1 mM $MgCl_2$, 500 mM KCl, 1 mM $CaCl_2$, 5 mM puromycin, and 100 U/ml staphylococcal nuclease) or LSB. Each membrane suspension was then incubated on ice for 30 min followed by a further incubation at 37°C for 15 min. Samples were then layered onto continuous 30–48% (wt/wt) sucrose gradients, supported by a 65% (wt/wt) sucrose cushion, and centrifuged in an SW 41 rotor (Beckman Instruments, Inc.) at 40,000 rpm for 18 h, at 4°C. To observe the effect of ribosome removal on translocation-competent membranes, 200 mU of ribophorin I-containing membranes were used in an in vitro cotranslational translocation assay before being dispersed into dissociation buffer or LSB and processed as above.

Isolation of Density-shifted Membranes. Membrane-associated ribosomes were removed from crude RER membranes (obtained by fractionation along 30–48% wt/wt sucrose gradients as described above) according to the method of Kreibich et al. (19) with the following modifications. Rough membranes were resuspended in HSB (50 mM Tris, 500 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25 M sucrose) containing 100 U/ml staphylococcal nuclease and 5 mM puromycin, at a density of 60 OD U/ml and incubated for 40 min at 0°C. Membranes were then dispersed in 2 vol of 71% wt/wt sucrose in TKM, overlaid with 48 and 30% wt/wt sucrose in TKM, and centrifuged at 50,000 rpm, for 2.5 h at 4°C in an SW50.1 rotor (Beckman Instruments, Inc.). Treated membranes were recovered from the 48/30% interface, pelleted, and resuspended by light homogenization in HSB. The A_{260}/A_{280} ratio was determined before further addition of puromycin or nuclease. Membranes were recycled through this treatment until a A_{260}/A_{280} ratio of 1.0 was obtained. Fully stripped membranes were then dispersed in 71% wt/wt sucrose TKM overlaid with 48, 39, and 30% wt/wt sucrose in TKM and centrifuged in an SW50.1 rotor (Beckman Instruments, Inc.) at 50,000 rpm for 25 h at 4°C. Density-shifted membranes were collected from the 39/30% interface, while nonshifted membranes were collected from the 48/39% interface.

Analyses of Membrane Association

Floatation analysis was performed by dispersing crude microsomal membranes into 2 ml of 71% wt/wt sucrose TKM overlaid with 2 ml each of 51 and 30% wt/wt sucrose TKM and centrifuging in a SW50.1 rotor (Beckman Instruments, Inc.) at 50,000 rpm, 4°C for 2.5 h. Membranes were collected from the 30/51% interface. Material remaining in the starting fraction, or sedimented during this procedure was collected by combining the 51/71% interface, the 71% fraction, and the pellet. Each fraction was diluted by the addition of 3 vol of TKM. Analysis of the fractions was carried out by SDS-PAGE and fluorography of TCA-precipitated aliquots of these fractions.

Translocation-competent membranes, prepared as described by Rothblatt and Meyer (30), were treated with Na_2CO_3 at pH 11.5 according to the method of Hortsch et al. (16). Triton X-114 detergent solubilization and cloud-point precipitation of membranes was performed by the method of Bordier (6).

Proteinase K Treatment

Proteolysis was performed at 0°C for 90 min, using proteinase K at a final concentration of 0.4 or 1.2 mg/ml, either in the absence or presence of 0.5% Triton X-100, 500 mM NaCl as indicated in the figure legends. Proteolysis was stopped by the addition of PMSF to a final concentration of 1.3 mg/ml for 5 min at 0°C.

Analysis of Glycosylation

Endoglycosidase H digestion was performed on crude microsomes (solubilized in 0.1% SDS, 100 mM citrate/phosphate buffer, pH 5.0, 25 mM DTT) at a final concentration of 10 A_{280} U/ml. Endoglycosidase H was then added to a final concentration of 100 mU/ml along with a protease inhibitor cocktail containing 0.2 mg/ml PMSF, 1 mg/ml IAA, and leupeptin, chymostatin, and pepstatin at final concentrations of 1 μg/ml. Digestions were performed at 37°C for the times indicated. Reactions were stopped by the addition of an equal volume of SDS-PAGE sample buffer followed by heating at 95°C for 5 min. Con A affinity chromatography was performed as described by Baker et al. (3).

Transmission EM

Membrane fractions were harvested by centrifugation at 16,000 rpm, for 15 min in a microfuge B (Beckman Instruments, Inc.). Pellets were fixed in mixed aldehydes consisting of 1.5% formaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h at room temperature. Membranes were then repelleted and postfixed for 2 h in 2% O_8O_4, 0.1 M cacodylate buffer, pH 7.2, before counterstaining en bloc with 1% uranyl acetate for 16 h. Samples were then taken through serial dehydration in ethanol and embedded according to the method of Spurr (33).

Analytical Methods

PAGE and fluorography, were carried out on 10–15% gradient gels as de-

scribed by Blobel and Dobberstein (5). Silver staining was according to the method of Ansorge (2).

Results

Expression of Ribophorin I in Yeast

A full-length cDNA encoding human ribophorin I, cloned previously in our lab (7), was introduced into pEMBLyex4, placing the expression of the protein under control of the *GAL10* promoter. This enabled high levels of expression of ribophorin I mRNA when cells were grown in the presence of 2% galactose. Preliminary experiments showed that the 5′ untranslated region of the human clone was inhibitory to its translation in yeast. Accordingly, we undertook site-directed mutagenesis to remove 5′ untranslated sequences derived from ribophorin I cDNA, placing the vector-specific yeast cytochrome C_1 sequences immediately upstream from the ATG of ribophorin I. As can be seen in Fig. 1, the construct encoding human ribophorin I was efficiently transcribed and translated in yeast. Northern blotting analysis revealed a single mRNA species of ~2,500 bp in length, that could be detected with probes derived from the human cDNA clone (Fig. 1 *A*, lane *2*). Northern blots of RNA from control cells transfected with vector alone were negative (Fig. 1 *A*, lane *1*). Detection of ribophorin I expression using immunoblotting with an anticanine ribophorin I antibody, decorated a single species with an M_r in SDS of 65 kD in yeast cells transfected with the human gene (Fig. 1 *B*, lane *1*), whereas no signal was observed in vector-alone transfectants (Fig. 1 *B*, lane *2*). These results are consistent with the expression of an intact human ribophorin I molecule (16).

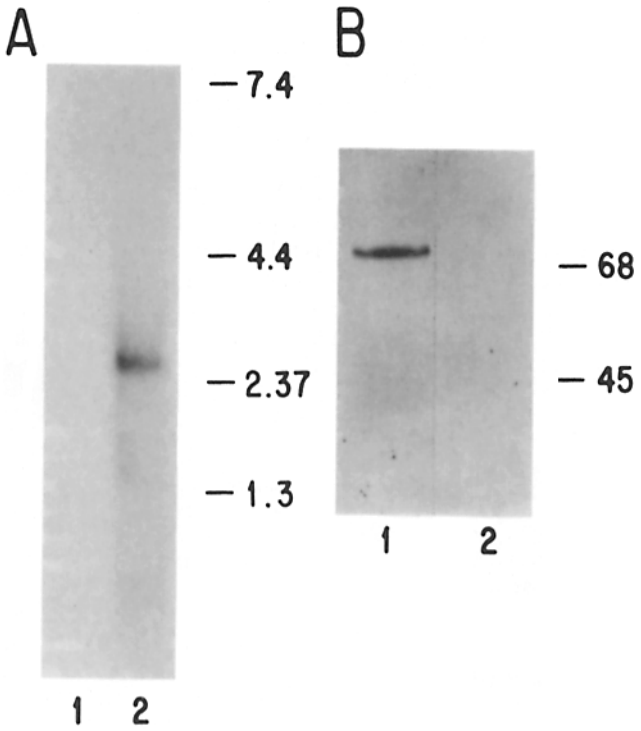

Figure 1. Expression of human ribophorin I in yeast cells. (*A*) Northern analysis using a human ribophorin I cDNA probe. Standards (in kbp) are shown on the right. (lane *1*) RNA from cell transfected with vector alone; (lane *2*) RNA from cells transfected with ribophorin I gene. (*B*) Immunoblots of yeast microsomal membranes obtained from cells transformed with ribophorin I cDNA (lane *1*) or vector alone (lane *2*) and decorated with a monoclonal anticanine ribophorin I.

Expressed Ribophorin I Is an Integral Membrane Protein

The correct targeting and assembly of ribophorin I into yeast RER was determined by a series of experiments. Human ribophorin I is an integral membrane glycoprotein comprising 584 amino acids, and is synthesized with a cleavable signal sequence of 23 amino acids (7, 29). It spans the membrane of the RER once with its NH_2-terminus in the lumen of the ER and its 150 amino acid–long COOH-terminus in the cytosol (7, 13). It is glycosylated once (7, 13, 21) at amino acid 276. These data allow several testable predictions, all of which must be found to be true to demonstrate correct assembly into and topology within yeast RER.

The presence of the protein in a membrane fraction can be demonstrated in three ways. Layering a yeast lysate below a sucrose gradient and allowing the membranes to float during centrifugation should show ribophorin I to be localized to a fraction that entered the gradient. Resistance to carbonate (pH 11) extraction and appearance in clould-point precipitates of TX-114–solubilized membranes are hallmarks of integral membrane proteins. Ribophorin I, if correctly integrated, should have these properties.

To show membrane association by floatation, a yeast microsomal fraction was dispersed into 71% sucrose and overlaid with steps of 51 and 30% sucrose. After centrifugation (see Materials and Methods), membranes were recovered from the 30/51% interface (referred to as "float" in Fig. 2), while material not associated with membranes was recovered by pooling the 51/71% interface, the 71% step, and the pellet (referred to in Fig. 2 as "cush"). Western blotting (Fig. 2, lanes *1* and *2*) indicated that ribophorin I was localized exclusively in the floated, i.e., membranous, fraction. A control was performed in which the yeast microsomes were "loaded" with prepro-α-factor posttranslationally (30) before centrifugation. As can be seen in lanes *3* and *4*, all of the translocated, glycosylated pro-α-factor and some untranslocated, but membrane-bound (32), prepro-α-factor was associated with the floated material. The cushion contained no glycosylated pro-α-factor, rather, only prepro-α-factor. These data represent our initial evidence that the ribophorin I is associated with a membranous fraction that

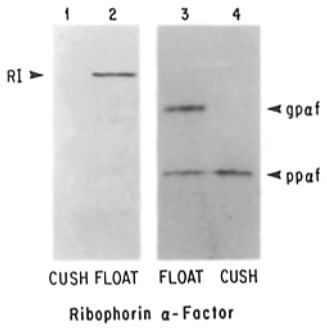

Figure 2. Expressed ribophorin I is membrane associated. Yeast microsomes, prepared from cells expressing human ribophorin I, were floated in sucrose gradients as described in Materials and Methods. Before centrifugation, membranes were incubated with a yeast lysate containing radiolabeled prepro-α-factor as the product of in vitro translation. Lanes *1* and *2* show an immunoblot stained with monoclonal anti-RI. Lanes *3* and *4* represent a fluorogram showing the relative distribution of prepro-α-factor (*ppαf*) and glycosylated pro-α-factor (*gpαf*).

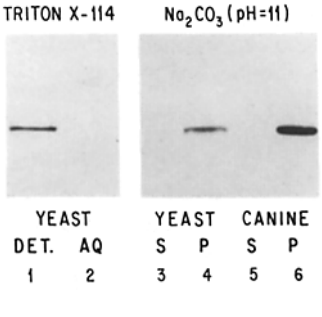

Figure 3. Expressed ribophorin I is an integral membrane protein. Yeast microsomes were solubilized in Triton X-100 and cloud point–precipitated, or washed in Na_2CO_3, pH 11, as described in Materials and Methods. Shown above are immunoblots decorated with monoclonal antiribophorin I. (lanes *1* and *2*) The detergent and aqueous phases, respectively, of a Triton X-114 cloud point precipitation. Lanes *3* and *5* represent the carbonate-soluble (*S*) and lanes *4* and *6* the carbonate-insoluble (*P*) material derived from treatment of yeast and canine microsomes, respectively.

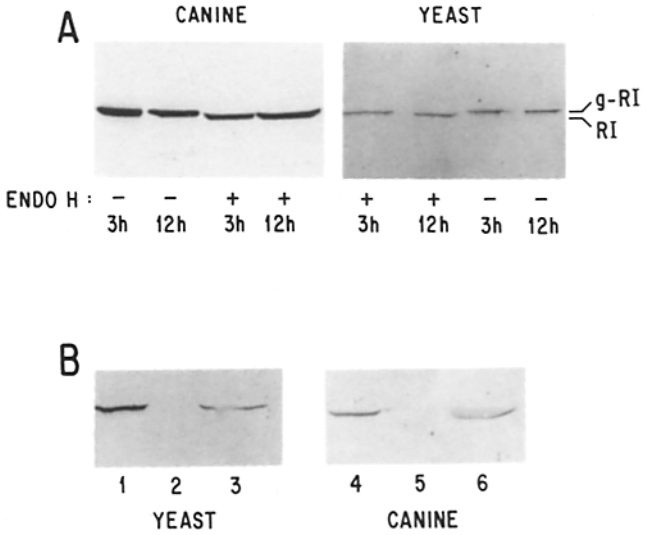

Figure 4. Expressed ribophorin I is glycosylated. Microsomes were solubilized and either digested with endoglycosidase H or chromatographed on columns of Con A as described in Materials and Methods. (*A*) Endo H treatment. Digestions were carried out for the times indicated. Shown are immunoblots stained with antiribophorin I. (On this gel system the shift from 65,000 D [glycosylated form] to 63,000 [deglycosylated form] is minimal but distinct.) (*B*) Con A chromatography. Lanes *1* and *4*, *2* and *5*, and *3* and *6* represent starting material, flow-through, and material bound by Con A Sepharose, respectively.

contains the translocation and glycosylation machinery of yeast RER.

To demonstrate membrane integration, microsomes containing expressed ribophorin I were isolated from yeast and subjected to extraction with 0.1 M Na_2CO_3. Canine pancreatic microsomes, a rich source of ribophorins, treated in the same way, served as a control. As can be seen in Fig. 3, lanes *3–6*, ribophorins were exclusively localized in the CO_3-resistant pellet fraction, typical of proteins that are integral to a membrane. Although more a function of the potential of a protein to be integral to a membrane than its actual topology, cloud-point precipitation of the detergent Triton X-114 was carried out in addition to carbonate extraction. When microsomes were solubilized in 1% TX-114 and subjected to precipitation temperatures (6), ribophorin I was exclusively recovered in the detergent phase, typical of membrane proteins (Fig. 3, lanes *1* and *2*).

Expressed Ribophorin I Assumes the Correct Topology

To determine if the topology of ribophorin I expressed in yeast was identical to that in mammalian cells, two types of experiments were carried out. As the primary sequence of ribophorin I indicates that it is glycosylated once on asparagine 276, correct assembly into the membrane would localize this NH_2-terminal domain in the lumen of the ER and result in its glycosylation. Correct topology is also characterized by a cytosolic disposition of the COOH-terminus of ribophorin I that would be accessible to exogenously added protease.

Two approaches were undertaken to ascertain glycosylation of ribophorin I expressed in yeast: (*a*) digestion of the proteins with endoglycosidase H; and (*b*) lectin-affinity chromatography on Con A. Shown in Fig. 4 are the results of these experiments. Although canine ribophorin I was capable of being digested more readily with endo H, hu-

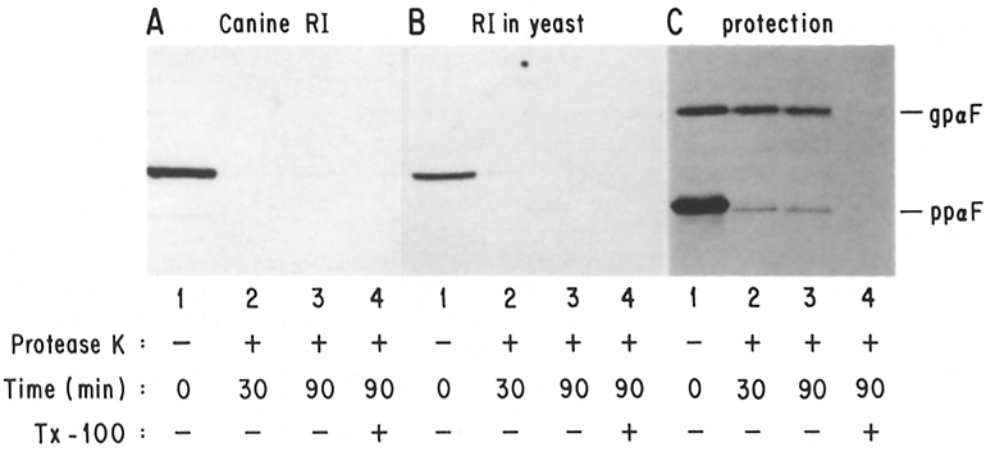

Figure 5. Expressed ribophorin I assumes the correct topology. Canine (as a control, *A*) and yeast (*B*) microsomes were incubated with proteinase K at 0°C for the periods indicated, in the presence or absence of Triton X-100. Immunoblots were decorated with a mAb that recognizes an epitope on the cytoplasmically exposed portion of ribophorin I. *C* represents the fluorogram of an identical assay performed on yeast microsomes that had been preincubated with yeast lysate containing radiolabeled prepro-α-factor as an in vitro translation product. This allows the translocated and glycosylated pro-α-factor to serve as a luminal control for assessing the intactness of the vesicles during the proteolysis. *ppαf*, prepro-α-factor; *gpαf*, glycosylated pro-α-factor.

man ribophorin I expressed in yeast was completely deglycosylated within 12 h of incubation with the glycosidase (Fig. 4 *A*). Although it is difficult to resolve a difference between 65 kD (glycosylated form) and 63 kD (deglycosylated form) on SDS gels, a clear difference was seen nonetheless. When ribophorin I was solubilized and passed over a Con A Sepharose column, both the human (from yeast) and the canine forms were efficiently bound by the lectin (*B*). Taken together, these data indicate that human ribophorin I is N-glycosylated once in yeast, and that the sugars are of the core type, typical of reactions taking place in the ER. It is worth noting that we never observed any outer chain mannose addition to ribophorin I that would be indicative of its transport to compartments further along the secretory pathway. These data represents the first suggestion that expressed ribophorin I is being retained in the ER.

To localize the COOH-terminus of ribophorin I to the cytosolic face of the membrane, yeast microsomes containing ribophorin I, and "loaded" with glycosylated pro-α-factor as a lumenal marker, were subjected to digestion with protease K. We made use of a mAb specific for an epitope contained within the COOH-terminal 150 amino acids (cytosolic domain) of the human ribophorin I. Thus, if the COOH-terminus is indeed cytosolically oriented, proteolysis should remove it, rendering the molecule undetectable with the specific antibody. The data shown in Fig. 5 demonstrate that this was indeed that case. After digestion of the yeast (or canine) microsomes with protease K, the ability to detect ribophorin I on Western blots was lost. The integrity of the microsomal membrane was maintained during this digestion as evidenced by protection of the glycosylated pro-α-factor in the lumen of the vesicles. Solubilization of the membranes with a detergent resulted in the digestion of the lumenal marker. From all of these studies we conclude that human ribophorin I is expressed in yeast as an integral membrane glycoprotein with the correct transmembrane topology.

Ribophorin I Is Retained in the RER

It is clear from the above data that ribophorin I is correctly assembled into the RER, however, the major issue to be resolved is its retention in this membrane system. If ribophorin I was not efficiently retained in RER, then it should have access to a smooth membrane compartment. The verifiable consequence of such transport would be a bimodal distribution of RI, as would be expected for nonspecific ER markers. This was addressed by fractionating membranes on gradients designed to separate rough and smooth membrane populations and carrying out a biochemical, morphological, and functional analysis of each.

Yeast microsomal fractions were loaded onto continuous 30–48% sucrose gradients and centrifuged at 160,000 *g* for 18 h. Fractions were collected and analyzed for protein, nucleic acids, BiP (the product of the yeast *KAR2* gene), prepro-α-factor translocation activity, and ribophorin I distribution. In addition, fractions were fixed, embedded, and thin sections prepared for EM. The data are shown in Figs. 6 and 7. The gradient centrifugation procedure resulted in the isolation of two distinct membrane populations (Fig. 6, *B*). The fraction of greater density (F_{II}) contained the highest amount of RNA (based on absorbance at 260 nm; Fig. 6 *C*), suggesting it was composed largely of rough, or ribosome-bearing membranes, while the lighter fraction (F_I) contained only smooth membranes. A morphological analysis of these two fractions by EM verified these indications (Fig. 7).

The yeast analogue of the heavy chain binding protein (BiP), a lumenal protein retained in the ER, was found to be present in both heavy and light fractions (Fig. 6, *D*). This is consistent with a generalized ER distribution, including both smooth and rough domains. Most importantly, the heavier (rough membrane) fraction possessed virtually all of the translocation activity as assayed by its ability to translocate and glycosylate prepro-α-factor in vitro (*E*). On immunoblots, it can be seen that all of the detectable ribophorin I was restricted to the heavier, translocation-competent fraction (*F*). From these data we conclude that ribophorin I is not only correctly targeted to and assembled into the RER, but is most likely retained there as well.

The dense membrane fraction (F_{II}) was evaluated by morphological criteria and found to consist of a heterologous population of membranes. It was thus necessary to determine if the expressed human Ribophorin I was contained and concentrated only within the rough membranes. To establish this we applied a density perturbation method based upon the chemical removal of ribosomes.

RER is unique insofar as it is the only organelle possessing large quantities of bound ribosomes. The ribosomes allow this organelle to have a significantly higher buoyant density (ρ_b) than would be predicted from the lipid to protein ratio of the membrane itself. Consequently, the chemical removal of ribosomes from RER should result in a shift in its ρ_b, enabling a separation from membranes with a similar densities. Strong supporting evidence of retention in the RER would be the observation that the ribophorin I would be shifted to a region of lower ρ_b as a result of chemical removal of ribosomes.

Accordingly, crude microsomal membranes containing ribophorin I were "stripped" of their ribosomes by treatment with a combination of nuclease, high salt, and puromycin (16) and fractionated on a 30–48% (continuous) sucrose gradient. For comparison "mock-stripped" crude microsomes were centrifuged on a comparable gradient (Fig. 8). The results clearly show that the equilibrium density of membranes containing ribophorin I underwent a shift from 1.19 to 1.17 g/ml as a result of ribosome removal. In addition, density-shifted membranes were further characterized with respect to translocation activity. Translocation-competent membranes that had been preloaded with glycosylated pro-α-factor in vitro, revealed a comparable shift in their buoyant density (Fig. 10). Comparison of the data presented in Figs. 8 and 10 shows that an almost identical density perturbation was observed for both ribophorin I-containing and translocation-competent membranes. A very similar shift was observed in the case of an ER enzymic marker, NADPH-cytochrome C oxidase, pursuant to the disruption of the ribosome membrane interaction by Mg^{2+} depletion (24).

We have determined that ribophorin I is concentrated in rough membranes, and that these same membranes are most highly enriched in the density-shifted fraction. This was accomplished by the use of sequential gradients (see Materials and Methods). The results presented in Fig. 9 show a significant enrichment of ribophorin I in density-shifted membranes (SHIFT). The striking contrast between the lev-

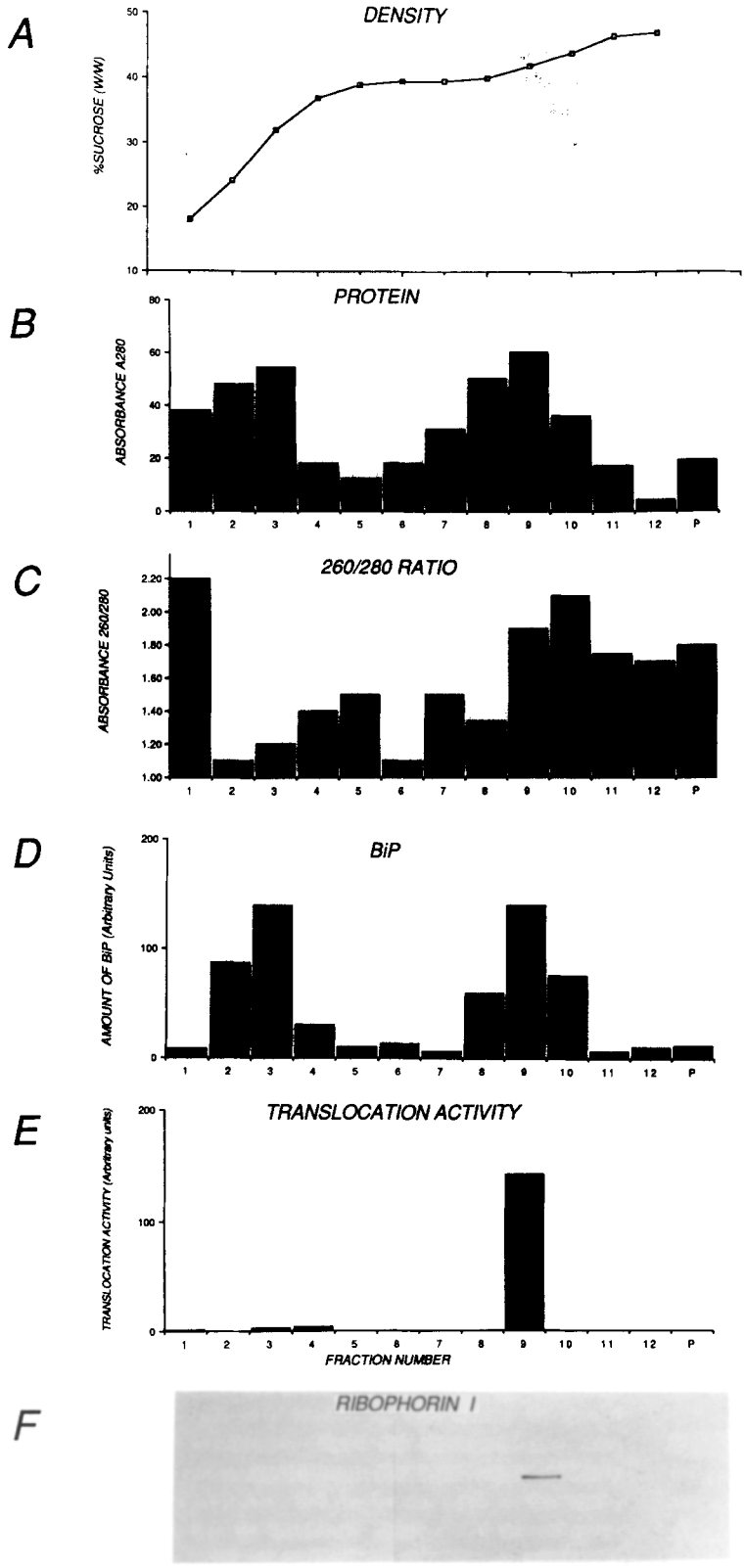

Figure 6. Ribophorin I is restricted to a dense membrane fraction. A crude yeast membrane fraction was applied to a 30–40% (wt/wt) sucrose gradient and centrifuged to equilibrium as described in Materials and Methods. (*A*) Sucrose density profile. (*B*) Distribution of protein. (*C*) A_{260}/A_{280} ratio of gradient fractions. (*D*) Distribution of yeast homologue of the heavy chain binding protein (*BiP*). (*E*) Distribution of translocation activity as evidence by the presence of glycosylated pro-α-factor (microsomal membranes were preloaded cotranslationally before centrifugation). (*F*) Distribution of ribophorin I as shown by immunoblotting of fractions with monoclonal antiribophorin I. All absorbance measurements were carried out in 2% SDS. When greater amounts of material were used for measurements shown in *E* and *F*, translocation activity and ribophorin I were observed in gradient fractions *8* and *10* as well.

els of ribophorin I in SHIFT versus nonshifted (NON) membranes is indicative of the specificity of the density perturbation procedure.

Fractionation of Yeast RER

In demonstrating that expressed human ribophorin I is retained in the RER of yeast cells, we have essentially worked out a scheme for the purification of fractions highly enriched in both morphological and functional attributes of this organelle (Sanderson, C. M., and D. I. Meyer, manuscript in preparation). The density-shifted membrane fraction that contained translocation activity (Fig. 10), was also charac-

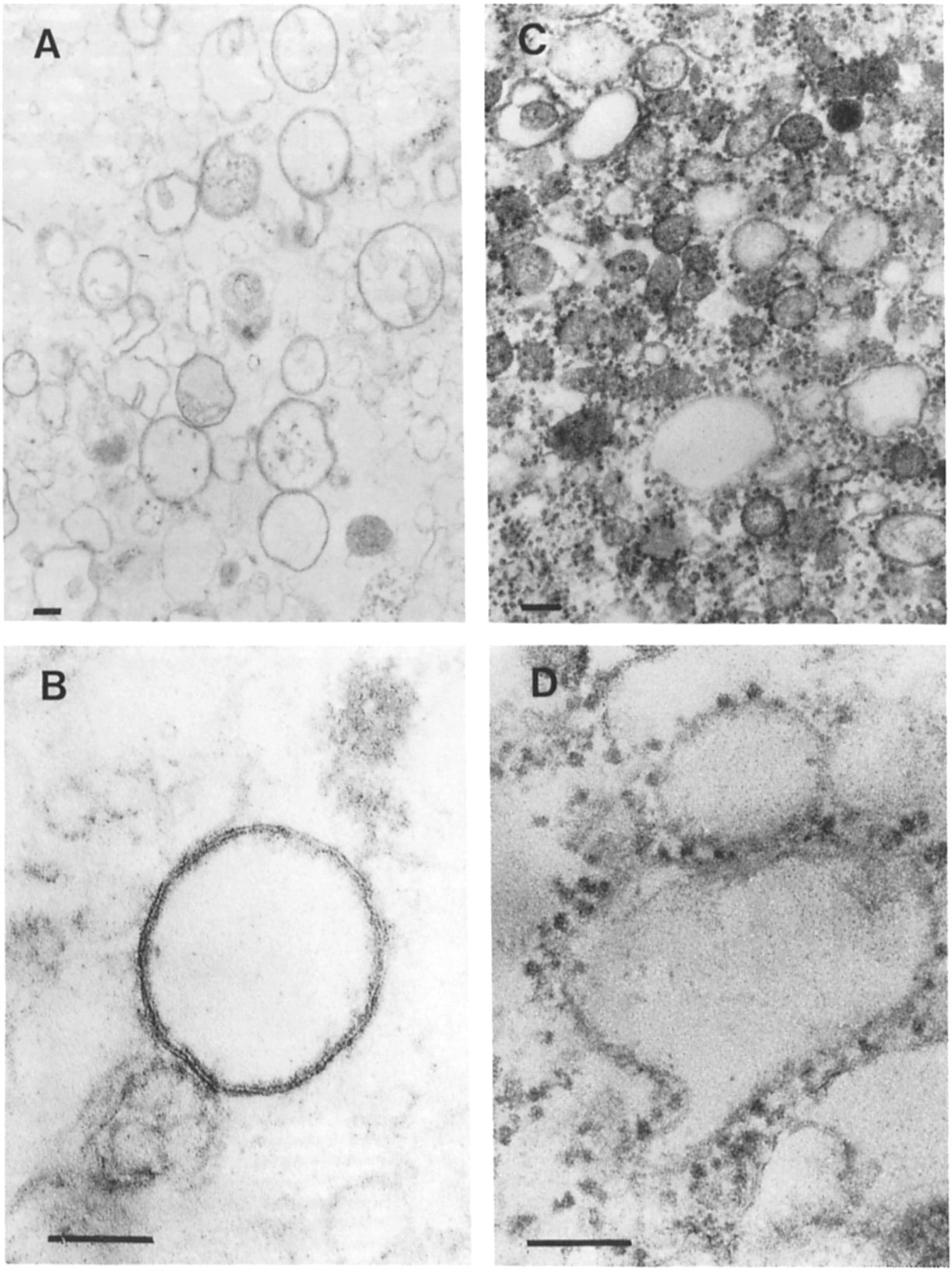

Figure 7. Morphology of membrane fractions after isopycnic centrifugation. Preparation of samples and EM were carried out as detailed in Materials and Methods. *A* and *B* depict membranes contained in fractions of lower density (F_I) at low and high magnification, respectively, whereas *C* and *D* show membranes derived from higher density fractions (F_{II}). Bars, 100 nm.

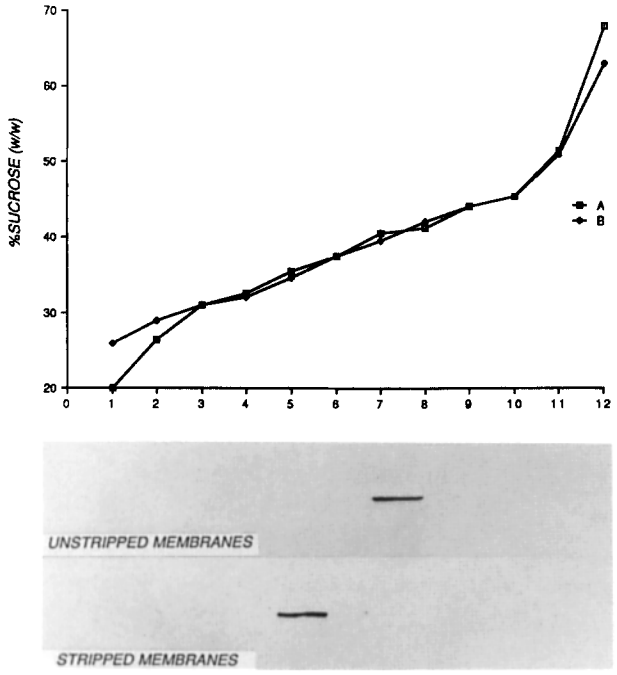

Figure 8. Removal of ribosomes results in a density shift of ribophorin I–containing membranes. Procedures are described in Materials and Methods. Shown here is the distribution of expressed human ribophorin I, as detected by immunoblotting, in mock-stripped (*upper*) and stripped (*lower*) membranes derived from F_{II}.

terized for its ability to carry out two previously identified subreactions of the translocation process. These included the binding of ribosomes in an in vitro assay (19), and the ability to bind secretory protein precursors, such as prepro-α-factor, in an ATP-independent fashion (32). These data imply that membrane fractions isolated in the way described above represent ideal starting material for biochemical analysis of secretory mutants as well as reconstitution of the translocation process.

Discussion

The data presented here indicate that a mammalian RER-specific protein, ribophorin I, is expressed, correctly targeted, assembled, processed, and retained in yeast RER. These findings are significant for two reasons. First, it establishes that the mechanism for protein retention in yeast RER is likely to be similar to that of mammalian cells. Second, a marker has been "planted" in yeast that could facilitate the isolation of RER in a highly purified form for further biochemical studies.

In mammalian cells, ER is clearly divided into two distinct domains: flattened, ribosome-studded cisternae; and a smooth-surfaced, mostly tubular network. Their relationship to one another has been documented both morphologically and biochemically. In liver, where it is particularly obvious, clear zones of continuity can be seen connecting rough and smooth elements (11). The protein composition of these two membrane systems shows a peculiar distribution

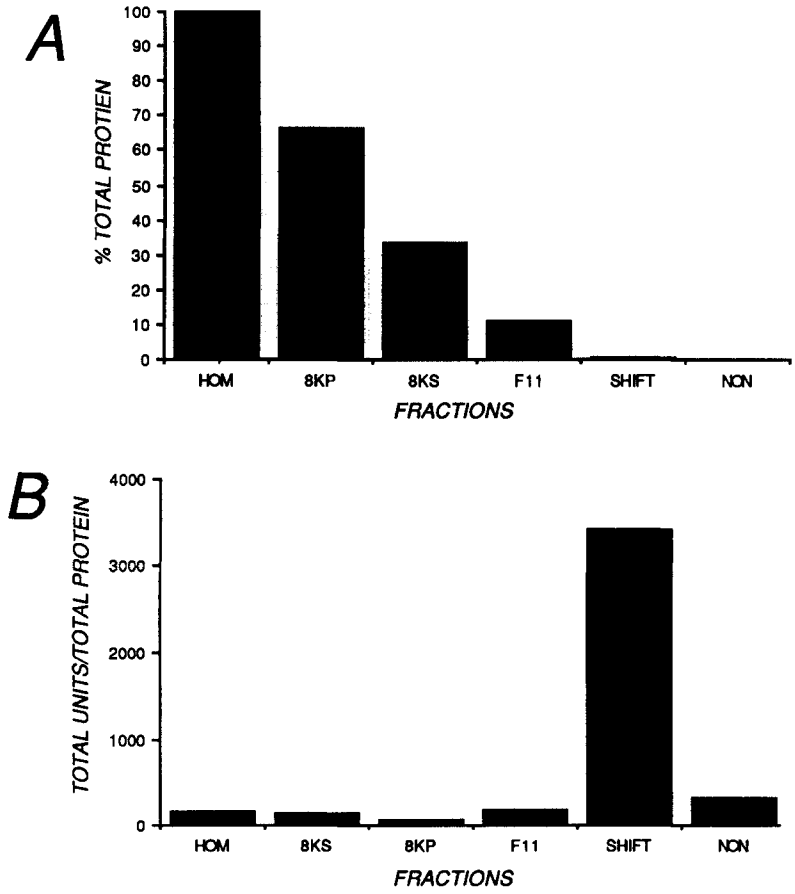

Figure 9. Ribophorin I expressed in yeast is specifically enriched in density-shifted membranes. A crude homogenate prepared from yeast expressing human ribophorin I was fractionated as described in Materials and Methods and the concentration of ribophorin I in each fraction determined. (*A*) The percent of total cellular protein in each fraction. (*B*) The specific enrichment of ribophorin I in each fraction. *HOM*, crude homogenate; *8KP* and *8KS*, the pellet and supernatant from an 11,000 *g* sedimentation, respectively; F_{II}, membranes obtained by gradient centrifugation having an equilibrium density of 1.19 g/ml; *SHIFT*, density-shifted membranes; *NON*, mock-shifted membranes.

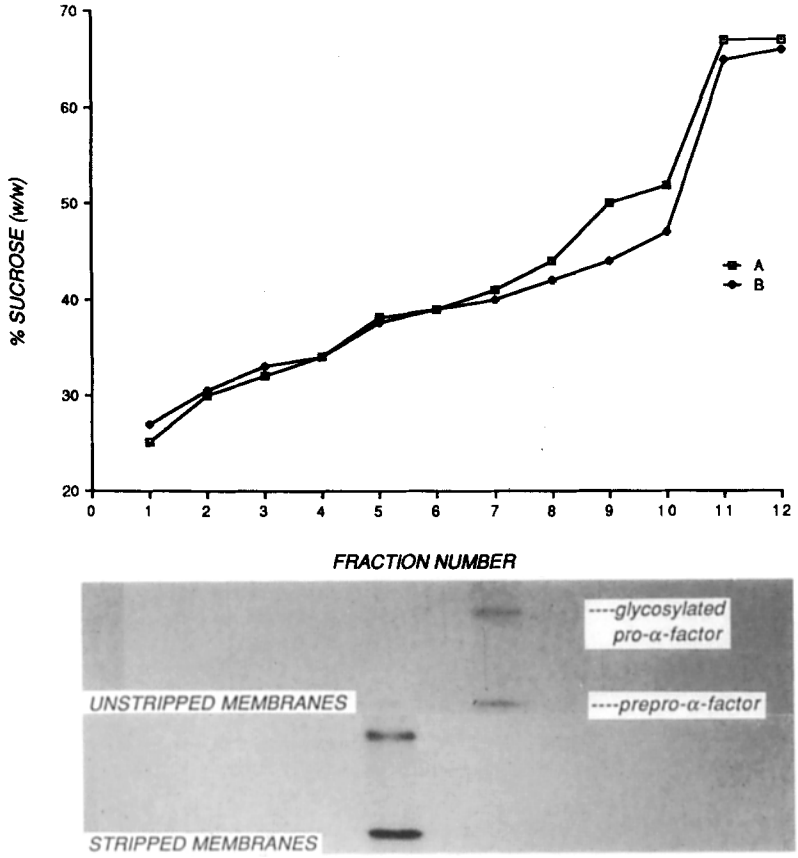

Figure 10. Removal of ribosomes results in a shift in the equilibrium density of translocation-competent membranes. Yeast microsomes were preloaded with radiolabeled prepro-α-factor in vitro before sucrose gradient centrifugation as described in Materials and Methods. The graph depicts the sucrose density of the individual fractions. *A* and *B* refer to gradients used for the sedimentation of mock-stripped and stripped membrane fractions, respectively. Shown below are autoradiograms indicating the distribution of translocation competence as evidenced by translocated and glycosylated prepro-α-factor.

of marker proteins. It appears that all proteins present in smooth ER are also contained, in significant proportions, in rough membranes (14, 19). This can be taken as biochemical support for the continuity of the two systems, in which certain proteins are free to diffuse between them. By contrast, RER has a unique set of proteins that rarely, if ever, have been detected in smooth ER (1, 19).

These two sets of proteins also seem to differ in their physical characteristics. Solubilization experiments have shown that RER-specific proteins are poorly soluble in nonionic detergents unless high concentrations of salt are included (16, 19). This implies that electrostatic interactions are involved in holding complexes of this type of membrane protein together. Indeed, immunochemical analysis has shown that RER proteins such as docking protein (SRP receptor), ribophorins I and II, and the signal peptidase complex are stubbornly insoluble in nonionic detergents unless concentrations of salt in excess of 250 mM are reached (10, 16). One can thus postulate that retention of proteins in the RER can occur by one of two mechanisms. Either there is a common retention signal, analogous to KDEL for luminal proteins, or retention occurs by the formation of protein complexes that are excluded from transport to smooth ER or to membranes further along the exocytic pathway.

In the case of certain viral membrane proteins, known to reside in organelles involved in early steps of the secretory pathway, specific sequences have been implicated in their retention (26). A particularly good example is that of the adenovirus E3/19K glycoprotein. This protein enables virus-infected cells to escape detection by the immune system by complexing MHC class I molecules in the ER and thwarting their transport to the cell surface. In this case, the motif of lysines at either −3 and −4 or the −3 and −5 positions from the COOH-terminus will retain chimeric membrane proteins in the ER. In addition to several forms of E3/19K, this motif exists in endogenous ER membrane markers such as UDP-glucuronyl transferase (17) and HMG-CoA reductase. Interestingly, yeast HMG CoA reductase does not exhibit this motif at its COOH-terminus (4).

Major differences between the cases of ribophorin I and E3/19K are worth noting. Foremost, is that neither ribophorin I, ribophorin II, nor docking protein, all shown to be restricted to the RER, possess this motif on their cytosolically disposed COOH-terminus. This implies that the E3/19K motif might be responsible for a generalized ER localization including elements of both smooth and rough ER. In fact, preliminary results seem to substantiate this interpretation (18). This is consistent with the KDEL motif for soluble proteins. KDEL-containing proteins such as BiP and protein disulfide isomerase are not localized specifically to either suborganelle. It would be most interesting to examine the distribution of E3/19K in the yeast system. If the aforementioned hypothesis were correct, E3/19K should exhibit a distribution similar to the yeast *KAR2* gene product, BiP (shown in Fig. 6).

The alternative mechanism for protein retention in RER may well lie in the ability of the specific proteins to form complexes with one another and in turn be either actively or passively restrained in their lateral translocation into smooth membranes. In either case, the expression of ribophorin I in

yeast can serve as an effective means for studying protein retention. Not only can a molecular genetic approach be taken in this organism for examining the structural requirements for retention, but the appropriate fusions when expressed in yeast could serve as the basis for a selection for retention mutants. For instance, a fusion of part or all of ribophorin I to invertase, when expressed in *SUC2*-deficient strains grown on sucrose, could lead to the isolation of ER membrane protein retention–defective cells. A similar approach has been used to isolate the *ERD* mutants that seem to be defective in retaining soluble proteins in the ER that possess an HDEL sequence on their COOH-terminus (12).

Ribophorin I expression did not have any deleterious effect on yeast metabolism and growth (data not shown). This makes it an ideal tool for all of the aforementioned studies, and for cell fractionation as well. It is for this reason that we have set out to use expressed ribophorin I as a marker to follow the purification of functionally active, highly enriched fractions of RER.

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